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Exhibit A

Mixed Backbone Oligonucleotides: Improvement in Oligonucleotide-Induced Toxicity *In Vivo*

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THE USE OF ANTISENSE OLIGONUCLEOTIDES as novel therapeutic agents has attracted attention over the last decade. A number of human clinical trials are underway using antisense oligonucleotides to treat infectious diseases, tumors, or immunologic disorders (reviewed in Akhtar and Agrawal, 1997). During the last few years, considerable advances have been made in understanding the issues regarding the mechanism of action, *in vivo* pharmacokinetics, metabolism, side effects, and toxicities of antisense oligonucleotides (Agrawal, 1996). These advances have enabled the design of new generations of antisense oligonucleotides. In this short report, we evaluate the toxicities of phosphorothioate oligonucleotides (PS-oligos), the first generation of antisense oligonucleotides, explore the rationale for designing mixed backbone oligonucleotides (MBO), the second generation of oligonucleotides, and compare their toxicities following systemic administration in mice.

PHOSPHOROTHIOATE OLIGONUCLEOTIDES AS FIRST-GENERATION OLIGONUCLEOTIDES

Phosphodiester oligonucleotides (PO-oligo) were initially studied, especially in cell-free systems and *in vitro* cell cultures. PO-oligos are not stable against nucleases and are, therefore, of limited use for *in vivo* applications. PS-oligos, in which one of the nonbridged oxygen atoms of the internucleotide linkage was replaced with a sulfur atom, were then developed to alleviate this problem. This modification provides PS-oligos with better stability against nucleases than PO-oligos. However, it also makes the backbone diastereomeric, and consequently, PS-oligos have lower binding affinities to their target mRNA sequences. The modification of PS-oligo also confers other property changes as well, as will be discussed here.

PS-oligos have been studied extensively in a numbers of disease models both *in vitro* and *in vivo* (Akhtar and Agrawal, 1997; Bennett, 1998). In many of these studies, PS-oligos have shown promising results, as evidenced by sequence-dependent antisense activities. There are some studies, however, in which PS-oligos have shown sequence-independent activities (e.g., Jansen et al., 1995), side effects, or toxicities (Sarmiento et al., 1994; Agrawal et al., 1997a; Henry et al., 1997b; reviewed in Agrawal, 1996). T xicity studies in mice and rats show thrombocytopenia, elevation of hepatic transaminases, hyperplasia, and infiltration of reticuloendothelial cells of various tissues (Sarmiento et al., 1994; Agrawal et al., 1997a; Henry et al., 1997b). The general toxicity profile after administration of PS-oligos of varying length or base composition or both is similar, but the severity of toxicity is quite different, suggesting that toxicity may be sequence dependent. In monkeys, complement activation and prolonged partial thromboplastin time (aPTT) have been observed following PS-oligo administration. These phenomena are not sequence dependent, suggesting that they may be due to the polyanionic nature of PS-oligos (Galbraith et al., 1994; Henry et al., 1997a; Shaw et al., 1997).

It has been shown that PS-oligos with certain sequences and structure motifs have immune stimulatory effects (reviewed in Pisetsky, 1996). Distinct patterns and kinetics of cytokine (IL-6, IL-12), chemokine (MIP-1β, MCP-1), and immunoglobulin production have been observed following *in vivo* administration of certain PS-oligos in mice (Zhao et al., 1997). Some cytokines (e.g., IL-6 and IL-12) can cause thrombocytopenia and elevation of hepatic transaminases in animals (Le and Vilcek, 1989). It is possible that the toxicity induced by PS-oligos is secondary to cytokine secretion. Of note, some cytokines (e.g., IL-12) and chemokines (e.g., MIP-1β) have shown antitumor or antiviral activities (Biron, 1994; Lusso et al., 1996). Thus, if a PS-oligo being studied as an antisense molecule for its antiviral or antitumor effects also has immune stimulatory effects, the impact of immune stimulation properties on its antitumor or antiviral effects needs to be carefully evaluated.

CONSIDERATIONS FOR SECOND-GENERATION OLIGONUCLEOTIDES

The usefulness of antisense oligonucleotides as therapeutic agents depends on many properties. From the studies carried out to date, it seems that the ideal oligonucleotide should (1) have specific and strong affinity for the target mRNA, (2) be stable against nucleases, (3) be taken up by target cells in sufficient amounts, and (4) not be toxic following *in vivo* administration. Oligonucleotide-induced toxicity is one of the most important parameters in considering the therapeutic potential of an antisense oligonucleotide. If a given antisense oligonucleotide shows toxicity following *in vivo* administration, its therapeutic window will be narrow, and its therapeutic usefulness may be

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limited. We have evaluated issues underlying the toxicity of PS-oligos and explored the possibility of how to minimize the toxicity with a new generation of oligonucleotides while maintaining their biologic activities.

MBO AS SECOND GENERATION OF OLIGONUCLEOTIDES

MBOs contain segments of PS-oligos and appropriately placed segments of modified oligodeoxynucleotides or oligoribonucleotides. The purpose of designing MBOs is to take advantage of the optimal properties of both the PS-oligo (e.g., RNase H activation) and the modified oligodeoxynucleotide (e.g., methyl phosphonate analogs provide good nuclease resistance and nonionic nature) or oligoribonucleotide (e.g., 2′-*O*-methyloligoribonucleotide phosphorothioate linkages provide good duplex stability and nuclease resistance). In MBOs, the choice of oligonucleotide modification and its placement in the oligonucleotide are crucial (Agrawal, 1996; Agrawal et al., 1997b). In this study, we used end-modified MBOs in which the segments of 2′-*O*-methylribonucleosides are placed at both the 3′-end and the 5′-end and the central region is PS-oligo.

OLIGONUCLEOTIDE DIRECTED TO RIα SUBUNIT OF PROTEIN KINASE A (PKA)

We studied the toxicities of two PS-oligos, oligo 1 (5′-CCG CTC TTC CTC ACT GGT-3′) and oligo 2 (5′-GCG TGC CTC CTC ACT GGC-3′), which are complementary to protein kinase A (PKA) regulatory subunit RIα (8–13 codons) of mouse and human mRNA, respectively (Nesterova and Cho-Chung, 195; Cho-Chung, 1997). Oligo 1 and oligo 2 share 100% ho-

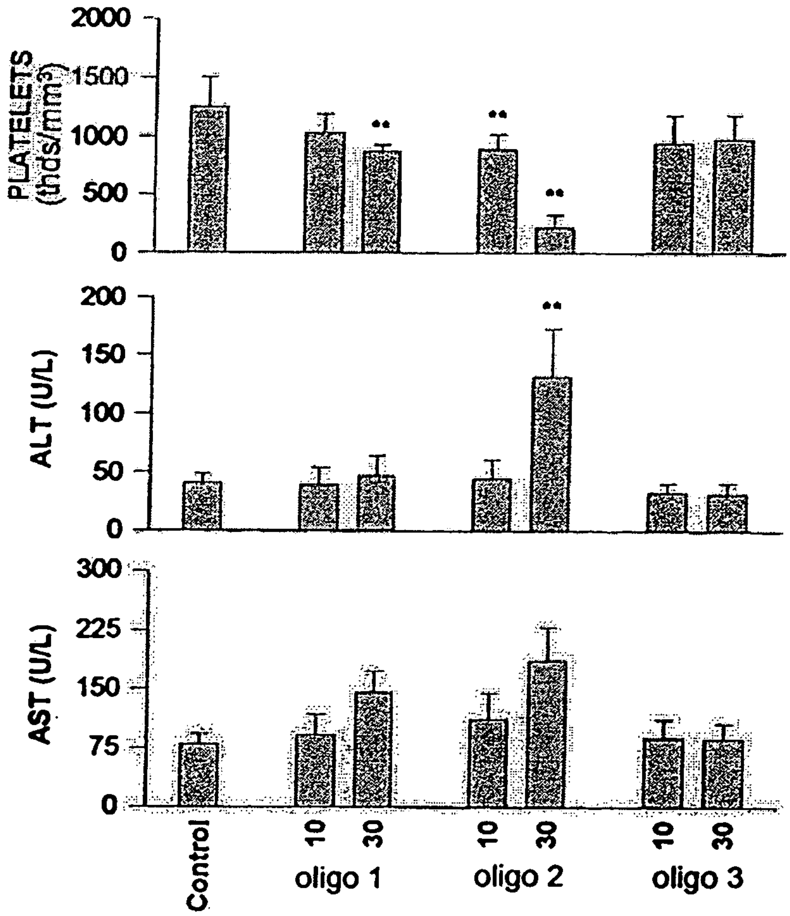

FIG. 1. The effects of oligonucleotides on hematologic and clinical chemistry parameters after 7 days of intravenous administration. Oligonucleotides were synthesized using standard phosphoramidite chemistry and purified by reversed phase HPLC. The identity of the oligonucleotides was checked by capillary gel electrophoresis. The purity of the oligonucleotides was 99%, with the percentage of the parent length greater than 90%. Oligonucleotides (oligo 1:5′-CCG CTC TTC CTC ACT GGT-3′, oligo 2: 5′-GCG TGC CTC CTC ACT GGC-3′, and oligo 3: 5′-GCGUGC-CTCCTCACUGGC-3′; sequences in bold refers to 2′-*O*-methylribonucleosides) were dissolved in sterile water and administered intravenously to male CD-1 mice (six per group) at either 10 or 30 mg/kg daily for 7 days. An equal volume of sterile water was administered in the same manner to control mice. Mice were killed on day 8, and peripheral blood was harvested. Mean platelet counts were measured using a Technicon H-1™ automated Hematology Analyzer, and serum ALT and AST levels were measured using a Roche Cobas Fara Chemistry Analyzer. Results were compared between groups by a one-way ANOVA test, and the mean ± SD of six mice for each group are presented. $^{*}p < 0.05$, $^{**}p < 0.01$.

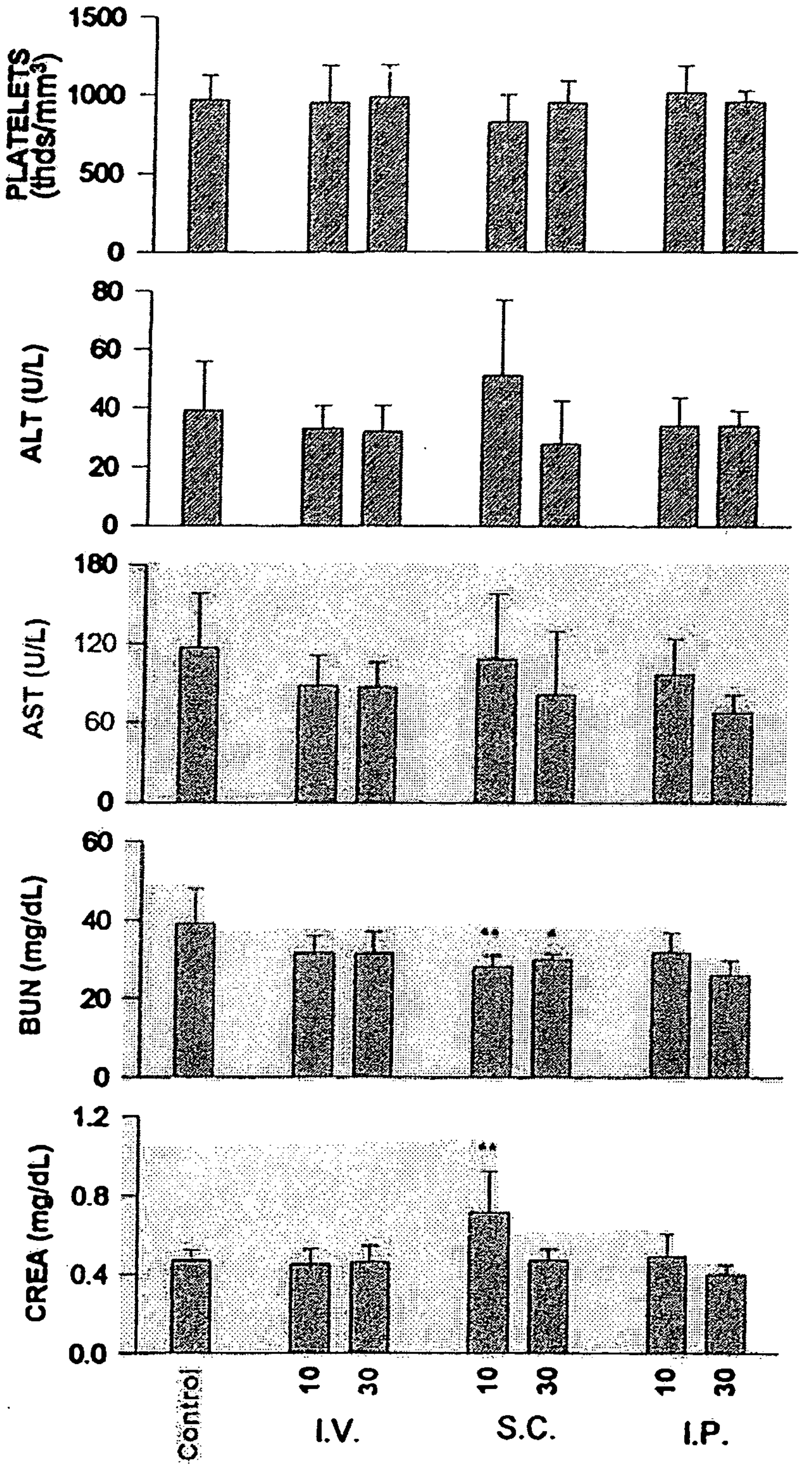

FIG. 2. The effects of oligonucleotide on hematologic and clinical parameters following 7 days of different routes of administration. Oligonucleotide (oligo 3) was dissolved in sterile water and administered intravenously (I.V.), subcutaneously (S.C.), or intraperitoneally (I.P.) to male CD-1 mice (six per group) at 10 or 30 mg/kg daily for 7 days. Mice were killed on day 8, and peripheral blood was harvested. Various hematologic and clinical chemistry parameters were measured in the same manner as described for Figure 1.

mology of 10 nucleotides: TCC TCA CTG GT. The toxicity studies were carried out in male CD-1 mice after 7 days of daily intravenous administration of the PS-oligos at 10 or 30 mg/kg. In general, no abnormal clinical signs were observed after oligonucleotide administration. Administration of 10 or 30 mg/kg of oligo 1 resulted in a reduction in mean platelet counts of 18% and 31% ($p < 0.01$), respectively, compared with the control mice. Mean serum alanine aminotransferase (ALT) and serum aspartate aminotransferase (AST) levels in mice that received 30 mg/kg of oligo 1 were 15% and 83%, respectively, greater than those of the control group (Fig. 1). Absolute spleen weights in mice that received 10 or 30 mg/kg oligo 1 were 19% and 59% higher than control values (data not shown).

The toxicities observed in mice that received oligo 2 were generally more severe than in mice that received oligo 1. The mean platelet counts in mice that received 10 or 30 mg/kg of oligo 2 were 29% ($p < 0.01$) and 82% ($p < 0.01$), respectively, less than those of the control group. Mice that received 30 mg/kg of oligo 2 had mean ALT and AST levels 222% ($p < 0.01$) and 134% ($p < 0.01$), respectively, higher than those of the control group. Absolute spleen weights in mice that received 10 or 30 mg/kg of oligo 2 were 33% and 87%, respectively, higher than the control values (data not shown). These differences in the severity of the toxicity profile further support the hypothesis that oligonucleotide-induced toxicity is primarily related to the sequence of the oligonucleotides rather than the length (both oligos are of 18-mer) or the target gene. Of note, although oligo 1 targets mouse PKA subunit R1α, it is unlikely that the toxicity observed here is due to downregulation of the target gene under the experimental conditions.

Our goal is to develop oligo 2 as an anticancer agent. Previous studies have shown that oligo 2 can downregulate the RIα subunit of PKA and inhibit the growth of tumor (LS-174T human colon carcinoma) in a tumor-bearing nude mouse model (Nesterova and Cho-Chung, 1995). The toxicity profile observed here suggests that oligo 2 shows toxicity in mice and may indicate a narrow therapeutic window. While analyzing the toxicity of PS-oligos in mice and rats using sequences of varying length and base composition, we found a strong correlation between toxicity and the immune stimulatory effects of the oligonucleotides (Agrawal et al., 1997). A CG dinucleotide with an optimal flanking sequence has been found to be closely associated with the immune stimulatory effects of oligonucleotides (Krieg et al., 1995). Appropriate modifications of the CG dinucleotide in an oligonucleotide can reduce the immune stimulation (Zhao et al., 1996) and the toxicity of PS-oligos in mice and rats (Agrawal et al., 1997a). To minimize the toxicity profile of oligo 2 without altering the oligonucleotide sequence, we substituted 2'-*O*-methylribonucleosides for four deoxynucleotides at both the 3'-end and the 5'-end (oligo 3). This substitution provided improvement in duplex affinity with target RNA and also increased nuclease stability (Agrawal, 1996). In addition, if the toxicity profile observed following administration of oligo 2 is the result of immune stimulation of the CG dinucleotide located near the 5'-end of the sequence, substitution of 2'-*O*-methylribonucleosides for the CG deoxynucleosides should minimize the immune stimulation effect, thereby reducing toxicity.

Oligo 3 was intravenously administered to CD-1 mice at doses f 10 or 30 mg/kg daily for 7 days. No adverse effects on body weight gain were observed. No statistically significant effects in clinical pathology parameters, including platelet counts, serum ALT, AST, blood urea nitrogen (BUN), and creatinine (CREA) levels, were observed following intravenous administration of 10 or 30 mg/kg of oligo 3 (Fig. 1). Mice were subjected to a limited gross necropsy examination of bone marrow of the femur, liver, kidneys, and spleen. No significant gross lesions were observed in these organs (data not shown). We also evaluated the toxicity of oligo 3 after subcutaneous or intraperitoneal administration. No significant abnormalities in mean platelet counts or ALT or AST levels were observed. Subcutaneous administration of 10 or 30 mg/kg of oligo 3 resulted in minor alterations in BUN and CREA levels. Decreases of 71%–76% in BUN levels in mice that received 10 or 30 mg/kg dose and an increase of 150% in CREA levels in mice that received 10 mg/kg dose were observed (Fig. 2).

The toxicity profile of oligo 3 is strikingly different from that of oligo 1, although both oligonucleotides have the same sequence. This observation supports the hypothesis that the toxicity induced by oligonucleotide is due to the immune stimulation. Chemical modification of the CG dinucleotide can minimize the immune stimulatory effects as well as the toxicity of oligonucleotides (Zhao et al., 1996; S. Agrawal et al., unpublished data). The antitumor effects of the chemically modified oligo, oligo 3, were maintained in a tumor-bearing nude mice model (data not shown).

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REFERENCES

AGRAWAL, S. (1996). Antisense oligonucleotides: Towards clinical trials Trends Biotechnol. 14, 376–387.

AGRAWAL, S., ZHAO, Q., JIANG, Z., OLIVER, C., GILES, H., HEATH, J., and SEROTA, D. (1997a). Toxicological effects of an oligodeoxynucleotide phosphorothioate and its analogs following intravenous administration in rats. Antisense Nucleic Acid Drug Dev. 7, 575–584.

AGRAWAL, S., JIANG, Z., ZHAO, Q., SHAW, D., CAI, Q., ROSKEY, A., CHANNAVAJJALA, L., SAXINGER, C., and ZHANG, R. (1997b). Mixed-backbone oligonucleotides as second generation antisense oligonucleotides: in vitro and in vivo studies. Proc. Nat. Acad. Sci. USA 94, 2620–2625.

AKHTAR, S., and AGRAWAL, S. (1997). *In vivo* studies with antisense oligonucleotide. Trends Pharm Sci. 18, 12–18.

BENNETT, C.F. (1998). Antisense oligonucleotides: Is the glass half full or half empty? Biochem. Pharmacol. 55, 9–19.

BIRON, C.A. (1994). Cytokines in the generation of immune responses and resolution of virus infection. Crit. Rev. Immunol. 6, 530–538.

CHO-CHUNG, Y.S. (1997). Antisense-protein kinase A: A single-gene-based therapeutic approach. Antisense Nucleic Acid Drug Dev. 7, 495–502.

GALBRAITH, W.M., HOBSON, W.C., GICLAS, P.C., SCHECHTER, P.J., and AGRAWAL, S. (1994). Complement acti-

vation and hemodynamic changes following intravenous administration of phosphorothioate oligonucleotides in the monkey. Antisense Res. Dev. 4, 201–206.

HENRY, S.P., GICLAS, P.C., LEEDS, J., PANGBURN, M., AULETTA, C., LEVIN, A.A., and KORNBRUST, D.J. (1997a). Activation of the alternative pathway of complement by a phosphorothioate oligonucleotide: Potential mechanism of action. J. Pharmacol. Exp. Ther. 281, 810–816.

HENRY, S.P., ZUCKERMAN, J.E., ROJKO, J., HALL, W.C., HARMAN, R.J., KITCHEN, D., and CROOKE, S.T. (1997b). Toxicological properties of several novel oligonucleotide analogs in mice. Anti-Cancer Drug Design 12, 1–14.

JANSEN, B., WADL, H., INOUE, S.A., TRULZSCH, B., SELZER, E., DUCHENE, M., EICHLER, H., WOLFF, K., and PEHAMBERGER, H. (1995). Phosphorothioate oligonucleotides reduce melanoma growth in a SCID-hu mouse model by a non-antisense mechanism. Antisense Res. Dev. 5, 271–277.

KRIEG, A.M., YI, A.E., MATSON, S., WALDSCHMIDT, T.J., BISHOP, G.A., TEASDALE, R., KORETZKY, G.A., and KLINMAN, D.M. (1995). CpG motifs in bacterial DNA trigger direct B-cell activation. Nature 374, 546–549.

LE, J.M., and VILCEK, J. (1989). Interleukin 6: A multifunctional cytokine regulating immune reactions and the acute phase protein response. Lab. Invest. 61, 588–602.

LUSSO, P., and GALLO, R.C. (1996). Role of β-chemokines in suppressing HIV replication. Science 274, 1393–1395.

NESTEROVA, M., and CHO-CHUNG, Y.S. (1995). A single-injection protein kinase A-directed antisense treatment to inhibit tumor growth. Nature Med 1, 528–533.

PISETSKY, D.S. (1996). Immune activation by bacterial DNA: A new genetic code [Review]. Immunity 5, 303–310, and references therein.

SARMIENTO, U.M., PEREZ, J.R., BECKER, J.M., and NARAYANA, R. (1994). *In vivo* toxicological effects of *rel* A antisense phosphorothioates in CD-1 mice. Antisense Res. Dev. 4, 99–107.

SHAW, D.R., RUSTAGI, P.K., KANDIMALLA, E.R., MANNING, A.N., JIANG, Z., and AGRAWAL, S. (1997). Effects of synthetic oligonucleotides on human complement and coagulation. Biochem. Pharmacol. 53, 1123–1132.

ZHAO, Q., TEMSAMANI, J., IADAROLA, P.L., JIANG, Z.W., and AGRAWAL, S. (1996). Effect of different chemical modified oligodeoxynucleotides on immune stimulation. Biochem. Pharmacol. 51, 173–182.

ZHAO, Q., TEMSAMANI, J., ZHOU, R.Z., and AGRAWAL, S. (1997). Pattern and kinetics of cytokine production following administration of phosphorothioate oligonucleotides in mice. Antisense Nucleic Acid Drug Dev. 7, 495–502.

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